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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GRIERSON, Donald [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB). LOWE, Alexandra, Louise [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB). HAMILTON, Andrew, John [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

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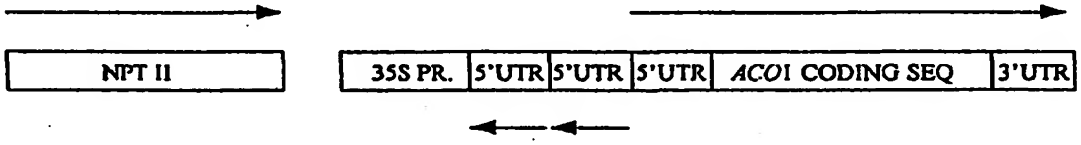


(57) Abstract

Constructs and methods for enhancing the inhibition of a target gene within an organism involve inserting into the gene silencing vector an inverted repeat sequence of all or part of a polynucleotide region within the vector. The inverted repeat sequence may be a synthetic polynucleotide sequence or comprise a modified natural polynucleotide sequence.

GENE SILENCING

This invention relates to the control of gene expression, more particularly to the inhibition of expression, commonly referred to as "gene silencing".

Two principal methods for the modulation of gene expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation"(also, referred to as "cosuppression"). Both of these methods lead to an inhibition of expression of the target gene.

In antisense downregulation, a DNA which is complementary to all or part of an endogenous target gene is inserted into the genome in reverse orientation. While the mechanism has not been fully elucidated, one theory is that transcription of such an antisense gene produces mRNA which is complementary in sequence to the mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 42 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each

Gene control by any of the methods described requires insertion of the sense or antisense sequence, under control of appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

The most widely used method is *Agrobacterium*- mediated transformation, mainly for dicotyledonous species. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium tumefaciens*, or the related *Agrobacterium rhizogenes*, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by *Agrobacterium* in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

targeted for silencing may be any part of that endogenous gene which characterises it, for example, its promoter, its 5'-untranslated region, its coding sequence or its 3'untranslated region. We have also found that the vector used in this invention will silence the expression of the target gene and also any members of the gene family to which the targeted gene belongs.

Although the mechanism by which the invention operates is not fully understood, we believe that creation of an inverted repeat promotes the formation of a duplex DNA between the selected sequence and its inverted.

The inverted repeat may be positioned anywhere within the vector such as within the promoter region, the 5' untranslated region, the coding sequence or the 3' untranslated region. If the inverted repeat is based on a contiguous sequence within the promoter region, then it is preferred that the inverted repeat in located within the promoter region. If the inverted repeat is based on a contiguous sequence within the 5' untranslated region, then it is preferred that the inverted repeat is located within the 5' untranslated region. If the inverted repeat is based on a contiguous sequence within the coding region, then it is preferred that the inverted repeat is located within the coding region. If the inverted repeat is based on a contiguous sequence within the 3' untranslated region, then it is preferred that the inverted repeat is located within the 3' untranslated region.

The selected polynucleotide sequence and its inverted repeat may or may not be separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex. It is preferred however, that the chosen contiguous sequence and its inverted repeat are separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex.

It is further preferred that the inverted repeat is based on the 5' untranslated sequence. It is also preferred that the inverted repeat is positioned upstream of the coding sequence. It is further preferred that the inverted repeat is positioned between the 5' untranslated region and the coding sequence. It is further preferred that the 5' untranslated region and the inverted repeat are separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex.

Without wishing to be bound by any particular theory of how it may work, the following is a discussion of our invention. 96% of tomato plants transformed with an ACC-oxidase sense gene containing two additional, upstream inverted copies of its 5' untranslated region, exhibited substantially reduced ACC-oxidase activity compared to wild type plants. Only 15% of plants transformed with a similar construct, without the inverted repeat, had reduced ACC-oxidase activity. Both populations had similar average numbers of transgenes per plant. Treatment of tomato leaves with cycloheximide caused a strong, reproducible increase in the abundance of ACC-oxidase transcripts and was used in the study of suppression by ACC-oxidase sense transgenes in preference to wound induction used in previous studies. The relative abundance of unprocessed and processed ACC-oxidase transcripts in suppressed and non-suppressed plants was assayed by ribonuclease protection assays, providing an indirect measure of transcription and mRNA accumulation which did not rely upon assaying isolated nuclei. This analysis indicated that the suppression of ACO1 gene expression was mainly post-transcriptional. Using the same type of RPA assay similar results were obtained from plants containing suppressing polygalactronase-sense or ACO-antisense transgenes.

There are now numerous examples of the inactivation of homologous sequences in plants. The term "homology dependent gene silencing" (HDGS) best describes all of these although it should be noted that in most examples the "silencing" is not complete and a low level of gene expression remains. Throughout this specification we will use the classification most-recently outlined by Matzke and Matzke, Plant Physiol. 107: 679-685 (1995) in which different examples of HDGS were divided into three main groups; cis-inactivation, trans-inactivation, and sense-suppression. Down regulation by antisense genes bears many similarities to the last of these and has been suggested to operated by the same mechanism (Grierson et al, Trends Biotechnol. 9: 122-123 (1991)). Both sense and antisense transgenes have been widely used to reduce the expression of homologous endogenous genes in plants. Although the underlying mechanisms of HDGS remain obscure, this technology has found numerous applications not only in fundamental research but also in commercial biotechnology ventures and new food products are already on the market.

At present, obtaining a large number of strongly suppressed, transgenic lines is more a matter of luck than judgement. A positive correlation between the presence of repeated

This invention gives a striking increase in the frequency of HDGS following the inclusion of a short repeated region within a transgene. Expression of the target gene encoding the terminal ethylene biosynthetic enzyme ACC-oxidase, in tomato was suppressed by such constructs mainly post-transcriptionally. This was shown to be true for other examples of sense and antisense suppression in tomato. Cycloheximide was found to be a potent and reliable inducer of ACO gene expression but did not ameliorate the silencing.

The invention will now be described, by way of illustration, in the following Examples and with reference to the accompanying Figures of which:

Figure 1. (A) ACO1 gene silencing vector.

(B) ACO1 gene silencing vector containing tandem inverted repeats of the 5' untranslated region.

Figure 2. Illustrates the relative ACC-oxidase activity in both types of transgenic plant relative to wild type values where C = transgenic plants containing construct C (Figure 1A) and V = transgenic plants containing construct V (Figure 1B).

Figure 3. Tomato plant ACC Oxidase activity of transgenic transformants containing pHIR-ACO (as illustrated in SEQ ID No 10). The graph also includes C12ACO (overexpression control) an untrasformed wild type and TOM13 strong antisense gene silenced control.

Example 1.0

Construct V (Figure 1) was made in the following manner: 79 base pairs of the 5' untranslated region of the tomato ACO1 cDNA was amplified by PCR and two copies were ligated in tandem in the reverse orientation immediately upstream of the ACO1 cDNA which contains its own polyadenylation signal in its 3' untranslated region (construct C). Both were ligated downstream of the CaMV 35S promoter and then transferred to the binary vector, Bin19. Figure 1 shows the basic details of constructs "C" and "V". These were used to transform tomato plants (Ailsa Craig) by *Agrobacterium* mediated DNA transfer. 13 and

Example 1.2

It was considered possible that the repeated region in the transgene might have affected the number of transgenes which integrated into the genome and that this was the actual source of high frequency silencing. The PCR assay described above can be used to estimate the transgene copy number if the following assumptions are made:

1) that in any transgenic plants there was no variation in the number of endogenous ACO1 genes per genome;

2) that the amplification efficiency ratio (endogenous ACO1 DNA: transgenic ACO1 DNA) is constant;

3) the reaction is sampled at low DNA concentration to minimise product re-annealing. Since we were only concerned with estimating the number of transgenes in the two lines relative to each other and not absolute quantification of transgene copy number, we did not employ synthetic combinations of "transgene" and "endogenous gene" DNA as standards.

After 20 cycles of amplification, gel-electrophoresis, Southern blotting, and hybridisation with a radioactively labelled ACO1 cDNA, the signal from endogenous and transgenic ACO1 DNA was visualised and quantified by phoshorimaging. The average transgene: endogenous gene ratio for the C line was 0.96 and for the V line 1.08 indicating that the repeat region in the V construct does not cause more T-DNAs to integrate during transformation.

Example 1.3

ACO1 mRNA increased in abundance following wounding and/or treatment of leaves with cycloheximide but accumulation was approximately five times greater after treatment with cycloheximide than after mechanical wounding which we have previously used as a stimulus. Wounding of cycloheximide treated leaves failed to elicit a further increase in ACO1 mRNA amount. We found cycloheximide to be a more reproducible inducer of ACO1 mRNA accumulation than mechanical wounding and so have used it in preference to

data shows that in the absence of silencing, the abundance of the endogenous and transgenic RNAs are comparable.

Example 1.5

We chose to measure the abundance of unprocessed transcripts in total RNA extracts as a indirect measurement of transcription whilst simultaneously measuring the amount of processed mRNA. This was achieved using RNA probes transcribed from genomic sequences spanning introns in ribonuclease protection assays. Since the RNA analysed was from leaves frozen in liquid nitrogen and then extracted in strongly protein-denaturing conditions (phenol and detergent) there should have been little opportunity for any resetting of transcription during the process There was a greater abundance of mRNA following treatment with cycloheximide although the total amount of mRNA in the ACO-AS plants was reduced. In the ACO-sense line, V11, there was little or no increase in the mRNA signal. It is likely that this mRNA signal is mainly from the transgene which is transcribed by the 35S promoter which is not cycloheximide inducible. In contrast, the abundance of the primary transcript in all RNA samples increased following cycloheximide treatment. This RNA species originates only from the endogenous ACO1 gene since the transgene has no introns. In all cases the suppressing transgene had little or no effect upon the abundance of the primary transcript.

Example 1.6

Cycloheximide strongly stimulated the accumulation of both the ACO1 primary transcript and mature mRNA. Quantification of the signal from primary transcripts and mature ACO1 RNA in wild type leaves before and after treatment with cycloheximide showed that there was a 6 fold increase in the abundance of unprocessed ACO1 RNA but a 13 fold increase in the amount of processed ACO1 RNA. The abundance of transgenic ACO1 RNA (transcribed from the 35S promoter) in the C line also rose upon treatment with cycloheximide.

Example 1.7

produce silencing. Although the PCR assay used here is not absolutely quantitative, it does suggest that the average transgene dosage is about 2 implying that some of the lines exhibiting suppression have single insertions. In several of our lines, the suppression obtained is profound (Figure 2) which makes this strategy even more attractive to those interested in specifically switching off gene expression. There is one previous report of the deliberate combination of repetitive DNA with a reporter gene effecting increased HDGS: Lohuis et al., Plant Journal, 8, 919-932 (1995) inserted a copy of a randomly isolated repetitive genomic sequence (RPS) upstream of GUS reporter gene and found that this element increased the frequency of variegation of transgene expression. This is an example of cis-inactivation, probably acts at the transcriptional level, and the authors considered it to be distinct from co-suppression/sense-suppression phenomena. Interestingly, the RPS element did not increase the frequency of complete silencing of the transgene. In our example, although the level of suppression is severe in many lines, it is not possible to say whether the degree of suppression is equal in all cells expressing the target gene or if the repeat has simply greatly increased the proportion of cells experiencing suppression.

Example 1.8

Constructs and transformation

The tomato ACO1 cDNA, pTOM13 was released from its original cloning vector, pAT153, (Promega), creating pG31. pG31 was digested with EcoRI and the vector re-ligated to create pTRD. This removed the 5' end of the cDNA which contains approximately 90 base pairs of the 3'untranslated region in the antisense orientation at its 5' end which may have been introduced artefactually during the original cloning of the pTOM13 cDNA . The remaining ACO1 sequence was cut out from pTRD with EcoRI and HindIII and ligated into $pT_7.T_{3\alpha}18$ (BRL) digested by EcoRI and the ends filled in with Klenow enzyme. The 5' untranslated region of the ACO1 transcript (minus approximately 10 bases at the 5' end) was amplified with Taq polymerase from oligo dT-primed cDNA of wounded tomato leaves with the primers 5' CATTCATCTCTTCAATCTTTTG 3' (SEQ ID No.2) and 5' CTTAATTTCTTGGTAAAGTGTTTTCC 3' (SEQ ID NO.3). This DNA was rendered flush ended with T4 DNA polymerase and ligated with the filled in pTRF to create pMI1. This reconstituted the EcoRI site at the 5' end and yielded a translatable ACO1 cDNA

tissue. These values were subtracted from the values obtained from the bottles containing leaf discs.

Example 1.10

PCR analysis of transgenic plants

DNA was extracted from singles leaves of wild type plants, plants homozygous for a ACO-antisense gene, and those transformed with the constructs of pBC1 and pBM17. Leaves were frozen in liquid nitrogen, briefly ground in eppendorf tubes with a disposable pipette tip, ground further after the addition of 200µl DNA extraction buffer (1% laurylsarcosine, 0.8% CTAB, 0.8M NaCl, 0.02M EDTA, 0.2M Tris/HCl (pH8)), heated to 65°C for 15 minutes, extracted once with phenol/chloroform and the DNA precipitated from the aqueous phase by the addition of 0.6 volumes of isopropanol. The DNA was recovered by centrifugation, the pellets washed in 70% ethanol, dried and redissolved in 200ul, of TE buffer. 1ul of this was used as template for simultaneous PCR amplification of the endogenous ACO1 gene and the transgene using the primers ACO1.1 (ATGGAGAACTTCCCAATTATTAACTTGGAAAAG SEQ ID NO 4) and the ACO1.2 (CTAAGCACTTGCAATTGGATCACTTTCCAT SEQ ID NO 5) for 21 cycles of 30 seconds at 95°C, 30 seconds at 65°C and 1 minute at 72°C. Amplified DNA was separated by electrophoresis in a 0.8% agarose/1xTBE gel and blotted onto HybondN+ in 0.4M NaOH for 6 hours. To detect the amplified ACO sequences, the DNA on the filter was hybridised with random prime labelled ACO1 cDNA. The filter was washed in 0.2xSSPE/1%SDS at 65°C followed by phosphorimaging of the radioactive signal.

Example 1.11

Treatment of leaves with cycloheximide and mechanical wounding

Compound leaves were excised with a sharp scalpel blade and immediately placed under water solution of 50µl.ml^{-1} cycloheximide (Sigma). Another 3 cm of the stalk was cut from the branch under the solution and the assembly was then left in a laminar airflow for six hours to allow the cycloheximide to enter the leaves.

50μg of leaf RNA was mixed with an equal volume of denaturation/loading solution (50% formamide; 25mM sodium phosphate (pH6.5); 10mM EDTA; 6.2% formaldehyde; 200μg.ml^{-1} ethidium bromide) and separated by electrophoresis on a 25mM sodium phosphate (pH6.5) /3.7% formaldehyde /1.5% agarose gel in 10mM sodium phosphate (pH6.5)/3.7% formaldehyde with continuous buffer re-circulation. The separated RNA was blotted onto Genescreen (Dupont) hybridisation membrane in 10mM sodium phosphate (pH6.5). The autocrosslink setting on a Stratalinker (Stratagene) was used to covalently link the RNA to the filter. The filter was prehybridised and then hybridised with a 32P-random prime labelled ACO1 cDNA probe. The filter was washed in 0.2xSSPE/1%SDS at 65°C and then exposed to Kodak X-omat film between two intensifying screens at -70 for 24 hours. Subsequently the radioactivity in each band was measured by phophorimaging.

Example 1.13

Ribonuclease protection analysis

RNA was extracted from cycloheximide treated leaves and fruit described above.

RNA probes were transcribed with T7 RNA polymerase at 20°C with α-^{32}P UTP (400Ci. mmol^{-1}) as the sole source of UTP. After 1 hour incubation, RNAase-free DNAase was used to remove the template and the probe was further purified on 6%polyacrylamide/8M urea/1xTBE gels. The band containing the full length probe was visualised by autoradiography. The gel slice containing this RNA was excised and placed in 1ml probe elution buffer (0.5M ammonium acetate; 1mMEDTA; 0.2% SDS) for between 6 and 14 hours at 37°C. Typically, between 20m and 100μl of this would be co-precipitated with between 20 or 100μg of the RNA to be tested plus two yeast RNA controls. The precipitated RNAs were redissolved in 30μl hybridisation solution (80% formamide; 40mM PIPES/NaOH; 0.4M sodium acetate; 1mM EDTA pH should be 6.4) heated to 65°C for 10 minutes and hybridised at 42°C for between 2 to 14 hours. The longer hybridisation times were purely for convenience since we easily detected even rare transcripts after only 2 hours of hybridisation. 300μl of RNAase digestion buffer (5mM EDTA; 200mM sodium acetate; 10mM Tris/HCl. Final pH of solution should be 7.5) containing either RNAaseONE (Promega) or RNAase T1 (Ambion) was added to each tube except one containing yeast RNA which received RNAase digestion buffer without any ribonuclease. Incubation of the

(Stratagene). The blunt PCR fragment was ligated into the SmaI site downstream of the invert repeat structure of pHIR-SIN to create pSIN-HIR-ACO.

The plant expression cassette from pHIR-ACO was isolated using AgeI and ligated into the binary vector pVB6 AgeI site to create pHIR-ACO SEQ ID No 10. The insert was orientated using restriction analysis to ensure that all the ORF that will be active in the plant were unidirectional. pHIR-ACO was transformed into *A.tumafaciens* LBA4404: and this used to transform tomato cotyledons (*Lycopersicum esculentum* var Ailsa Craig). Plants were regenerated from callus.

Example 2.1

Identification of Transgenic Plants

DNA was extracted from single leaves and extracted as described previously. Plants containing the HIR-ACO T-DNA insert were identified by PCR using an internal TOM13 sense primer (5' GCTGGACTCAAGTTTCAAGCCAAAG 3'SEQ ID No 8) and a NOS 3'UTR (untranslated region) specific antisense primer (5'CCATCTCATAAATAACGTCATGC3' SEQ ID No 9)

Example 2.2

ACC-oxidase assays

ACC-oxidase activity was measured as the ability of plant tissue to convert exogenous 1-aminocyclopropane-1-1carboxylic acid (ACC) to ethylene. Small leaves were removed from shoots and wounded with a scalpel before being placed into a 2ml sealable vial, and left for 30minutes. The vials were then sealed and left for an hour at room temperature , after which the ethylene in the head space was measured by gas chromatography as described my Smith et al., 1986. Ethylene was also measured from wildtype, over-expressing (C12) and antisense down-regulated plant material.

ATTTTCGGTC CTAACAAGTG GTATCAGAGC CAGATTCAAT AATGGAGTCA GGTGTAGTGG 600

TTCGATAATC GATGATTGAA CCAAGTTAGA AAGAGGTGTT CATCTTGACG GGTGTAGTTC 660

TAGCCGCAAC CTTTTTGACA GTAATGAAGA TTTTGATGGA GAAATTGTTT CAGAGAGGTT 720

CTCTGTGTTG AGACATAAAT TTTGTAAAGG AGATTATGGA GAGGAGAAGC AAGTTGTTGA 780

AGATTAAGTA AAGAAGGTGG ACAAATCTAT TTTGTCAGAA ATTCAGGCCA AGGGGGAGAT 840

TTGTTGGGTT TTATTTGCCC TGATTTTTTA CCATAAATAG GTTTTCCTTT AAGGAAAAGG 900

TTTTGAATTG ACTATTCTTT TTTTGGTAGG AAAAGGTTTA GGATTCTATA AATAGAGGCA 960

TGTTCCTTCT AACTTAATTA GCATTCACAA TGTAGTTTTA AGGGCTTTGA GAGTTTTGGT 1020

TAGAGGGAGA ATTTGTGAAC CTCTCATGTA TTCCGAGTGA ATTGGTTGAG GTTGTTTCCC 1080

TCTGTATTTT GTACTCTCAT GTTTATAGTG GATTGCTCAT TTCCTTTGTG GACGTAGGTC 1140

GATTGACCGA ACCACGTTAA ATCTTTGTGT CTTTTGGTAT ATTTCTCGTT GTCTTCTTAC 1200

TCGTGGTCTT TCGAGGTTTG CTTTGCTAGC TTCCGCGTTT ACACCTGCTT ATTTGCGGTC 1260

CTAACAGAGT TCGATGGGTT GAATCTATAA AAAGAAAAAT ATACTCGTGA TTCACGATTA 1320

TTTATATGAA AATATAATAA ATATTGAATT TCCTTTGCTA TTTCTTATGT TTACGTCTTT 1380

ATATTTCAAA TTATTCCACC AATACTGACA AGCCCTAGGC CATCTCTAGG AAATTCATAC 1440

AATTTTTTTT TTGTTGTTAA CTAGTTAAAT TGGCAGCCTT AAAGATTATT GTAAAATTCA 1500

AGGCAACTTC CTCAAGTACT ACAACTACAT TGTAACATCC CAGTCAAAGT GTCCTAAAAT 1560

TTTATAAAAT TTGACACATG AAACAATAGC ACAATAAATT TTAGTACTAT TGCAGCCATG 1620

GCCCATAAGC CATCATGTAT TATAGTCAAA ATGGGTCCTT TTCCAATTTG TCTTGATCCC 1680

AAAATCCCTT TGTAGGTAAG ATGGTTCAAC AAGGAACTAT GACTCTTAAG GTAGACTTGG 1740

ACTCATAGAC TTGTCATAAC TCATAAAGAC TTGGAATATA ATAATTATTC ATTTAAATTA 1800

TAATTCTCTA CTTTAATATC TTCTACTATA AATACCCTTT CAAAGCCTCA TTATTTGTAC 1860

ATCAAACATT GATATTCATC TCTTCAATCT TTTGTATTCA CATATTCTAT TTATTCAATA 1920

CACTTAGGAA AACACTTTAC CAAGAAATTA AGATGGAGAA CTTCCCAATT ATTAACTTGG 1980

AAAAGCTCAA TGGAGATGAG AGAGCCAACA CCATGGAAAT GATCAAAGAT GCTTGTGAGA 2040

ATTGGGGCTT CTTTGAGGTA ATCATAAATT ACATAAACAT ATTAATATGT TTGTTTCAAT 2100

TTATCAGTCA TACTTTTCTC TGTTTTAAAA TTAATGTCAC TTTCAATATT TAATAATTCG 2160

CATGACATGT TTATAACACA ACAAGATATA GGTTACATTT TGATACATTA TATATAACTT 2220

CTGTCACACG ACTCAAAAGT CTTTCTTAAT TTCTTGAATT CAATGATCGA TCAAACTAAG 2280

ACACGTAAAA TGAAACGGGG AATAGTAATT CTGTTTGCTT ATGTGATCAT TGTAGTTGGT 2340

GAACCATGGA ATTCCACATG AAGTAATGGA CACAGTAGAG AAAATGACAA AGGGACATTA 2400

CAAGAAGTGC ATGGAACAGA GGTTTAAGGA ACTAGTGGCA AGTAAGGGAC TTGAGGCTGT 2460

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTTAATTTCT TGGTAAAGTG TTTTCC 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucliec acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGGAGAACT TCCCAATTAT TAACTTGGAA AAG 33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAAGCACTT GCAATTGGAT CACTTTCCAT 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid

(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTCATA AATAACGTCA TGC 23

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1949 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PHIR-ACO SEQ ID NO 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCGGTGAAT TCCCATGGAG TCAAAGATTC AAATAGAGGA CCTAACAGAA CTCGCCGTAA 60

AGACTGGCGA ACAGTTCATA CAGAGTCTCT TACGACTCAA TGACAAGAAG AAAATCTTCG 120

TCAACATGGT GGAGCACGAC ACGCTTGTCT ACTCCAAAAA TATCAAAGAT ACAGTCTCAG 180

AAGACCAAAG GGCAATTGAG ACTTTTCAAC AAAGGGTAAT ATCCGGAAAC CTCCTCGGAT 240

TCCATTGCCC AGCTATCTGT CACTTTATTG TGAAGATAGT GGAAAAGGAG GTGGCTCCTA 300

CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA GATGCCTCTG CCGACAGTGG 360

TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA AAAGAAGACG TTCCAACCAC 420

GTCTTCAAAG CAAGTGGATT GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC 480

CCACTATCCT TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC 540

AGGGTACCGC GGCACGGCCA GCCACGCCGC TGAGCCCGCA GTTTCTCGAG TTTCTGCGGG 600

CTCAGCGGCG TGGCTGGCCG TGCCGCCCAT GGGCGGCGGG GCTGCAGGAA TTCGATATCA 660

AGCTTATCGA TACCGTCGAC CTCGAGGGGG GGCCCGGTAC CGGATCCCCT GCACATGGAG 720

AACTTCCCAA TTATTAACTT GGAAAAGCTC AATGGAGATG AGAGAGCCAA CACCATGGAA 780

ATGATCAAAG ATGCTTGTGA GAATTGGGGC TTCTTTGAGT TGGTGAACCA TGGAATTCCA 840

CATGAAGTAA TGGACACAGT AGAGAAAATG ACAAAGGGAC ATTACAAGAA GTGCATGGAA 900

CAGAGGTTTA AGGAACTAGT GGCAAGTAAG GGACTTGAGG CTGTTCAAGC TGAGGTTACT 960

GATTTAGATT GGGAAAGCAC TTTCTTCTTG CGCCATCTTC CTACTTCTAA TATCTCTCAA 1020

(A) DESCRIPTION: /desc = "PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCGGGTACCG CGGCACGGCC AGCCACGCCG CTGAGCCCGC AGTTTCTCGA GGATGGGTTG 60

GCTCCATGGG CGGCG 75

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 13

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGCCGCCCAT GGAGCCAACC CATCCTCGAG AAACTGCGGG CTCAGCGGCG TGGCTGGCCG 60

TGCCGCGGTA CCCGC 75

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 14

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGGGCGCCGC TCGAGTTTCT GCGGGCTCAG CGGCGTGGCT GGCCGTGCCG CCCATGGCGC 60

ATCGGG 66

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs

CLAIMS

1. A vector for enhancing the inhibition of a selected target gene within an organism, comprising a gene silencing vector characterised in that the said gene silencing vector includes a inverted repeat of all or part of a polynucleotide region within the vector.

2. A vector as claimed in claim 1, in which the inverted repeat sequence is a synthetic polynucleotide sequence and its inverted repeat sequence.

3. A vector as claimed in claim 1, in which the inverted repeat sequence is an inverted repeat of all or part of the said gene silencing vector.

4. A vector as claimed in claim 3, in which the inverted repeat sequence is an inverted repeat of the 5'-untranslated region of the gene silencing vector.

5. A method as claimed in any of claims 1 to 4, in which the inverted repeat is separated from the polynucleotide region by a sequence of nucleotides.

6. A method of controlling the expression of a DNA sequence in a target organism, comprising inserting into the genome of said organism an enhanced gene silencing vector as claimed in any of claims 1 to 4.

7. A vector for enhanced gene silencing comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said construct includes an inverted repeat of a region of said construct.

8. A vector as claimed in claim 7 in which the inverted repeat is a fragment of the 5'-untranslated region of the said construct.

C V

FIGURE 1A

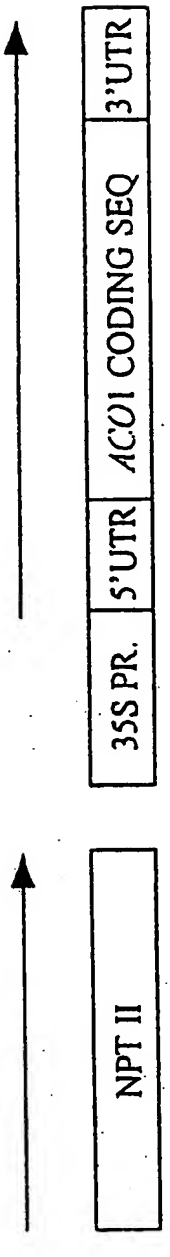


FIGURE 1B

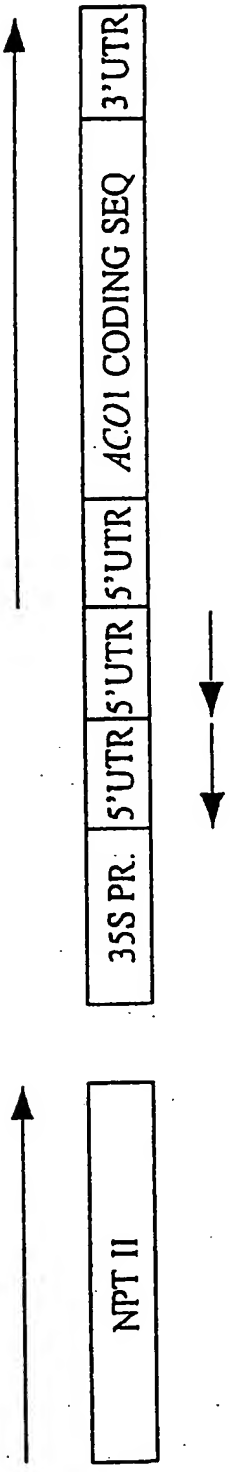
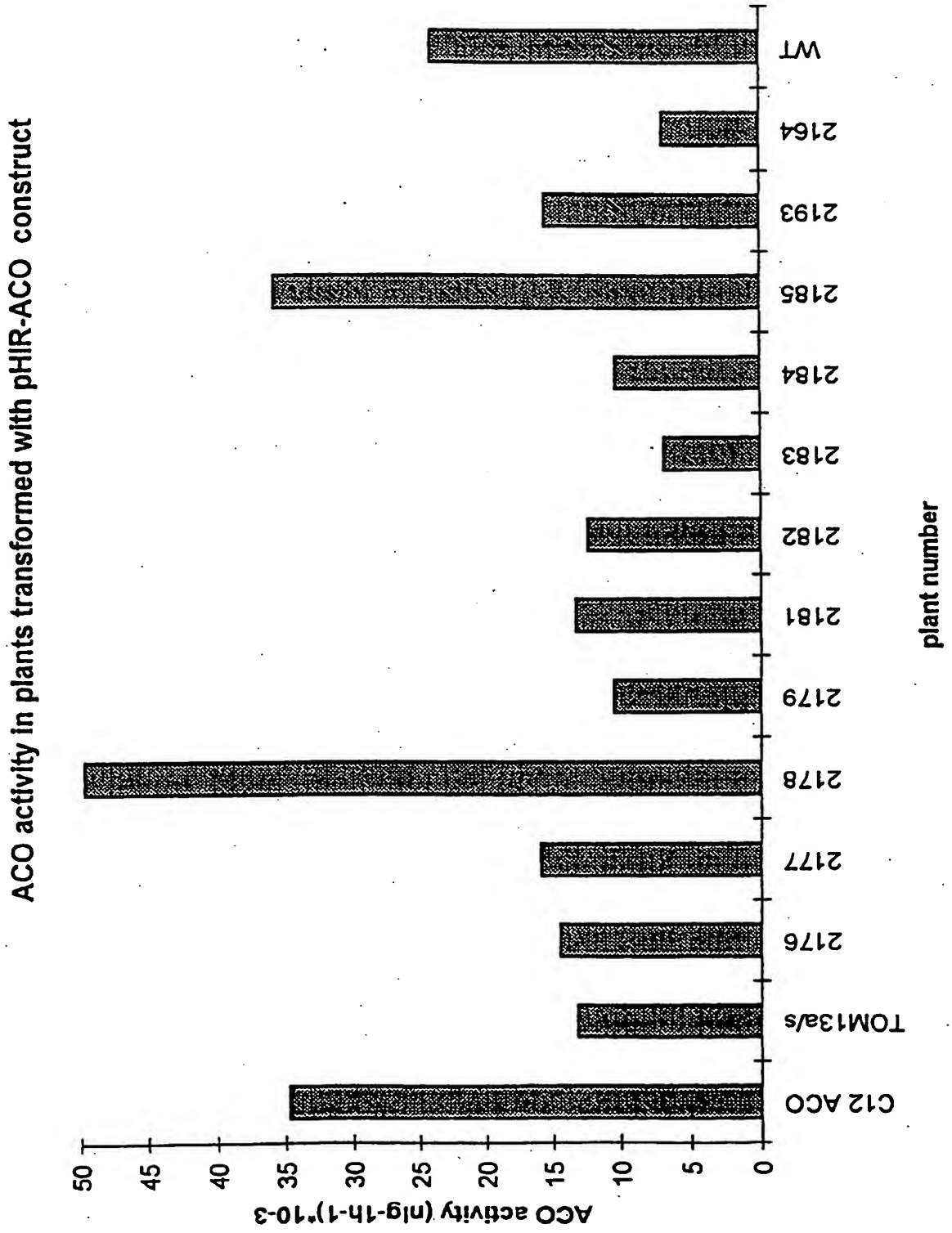


FIGURE 3


ACO activity in plants transformed with pHIR-ACO construct
ACO activity (nlg-1h-1)*10-3
0
5
10
15
20
25
30
35
40
45
50
C12 ACO
TOM13a/s
2176
2177
2178
2179
2181
2182
2183
2184
2185
2193
2164
WT
plant number

INTERNATIONAL SEARCH REPORT

National Application No
PCT/GB 98/01450

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TEN LOHUIS M. ET AL.: "A repetitive DNA fragment carrying a hot spot for de novo DNA methylation enhances expression variegation in tobacco and petunia" PLANT JOURNAL, vol. 8, no. 6, December 1995, pages 919-932, XP002075451 cited in the application see the whole document	1-12
A	WO 97 01952 A (DNA PLANT TECHN CORP) 23 January 1997 * see esp. p.13-15 *	1-12
A	WO 93 23551 A (SEYMOUR GRAHAM BARRON ;TUCKER GREGORY ALAN (GB); GRIERSON DONALD () 25 November 1993 see the whole document	1-12
A	GRIERSON, DON: "Silent genes and everlasting fruits and vegetables" NAT. BIOTECHNOL. (1996), 14(7), 828-829 CODEN: NABIF9;ISSN: 1087-0156, XP002075452 see the whole document	1-12
A	BLUME B ET AL: "Identification of transposon-like elements in non-coding regions of tomato ACC oxidase genes." MOLECULAR AND GENERAL GENETICS, (1997 APR 16) 254 (3) 297-303. JOURNAL CODE: NGP. ISSN: 0026-8925., XP002075453 see the whole document	1-12
A	HAMILTON, A. J. ET AL: "Post-transcriptional gene-silencing in tomato" MECH. APPL. GENE SILENCING, 'EASTER SCH. AGRIC. SCI.!, 57TH (1996), MEETING DATE 1995, 105-117. EDITOR(S): GRIERSON, DONALD;LYCETT, GRANTLEY W.; TUCKER, GREGORY A. PUBLISHER: NOTTINGHAM UNIVERSITY PRESS, NOTTINGHAM, UK. CODEN: 63NBAT, XP002075454 see the whole document	1-12
T	STAM, M. ET AL: "Post-transcriptional silencing of chalcone synthase in Petunia by inverted transgene repeats" PLANT J., (19970700) VOL. 12, NO. 1, PP. 63-82. ISSN: 0960-7412., XP002075455 see the whole document	1-12

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